Kinetics of Amino Acid Racemization (Epimerization) in the Dentine of Fossil and Modern Bear Teeth

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ABSTRACT: The present study examines the question of whether heating experiments on modern bear teeth dentine model the pattern of D/L racemization in fossil teeth. Using samples of modern bear teeth dentine heated at 65°C, 85°C (up to 53 days), and 105°C (up to 71 days), and three independently dated fossil bear teeth, we have compared the modes of racemization induced by temperature in the modern samples and by time on the fossil samples. We have studied seven amino acids (aspartic and glutamic acids, alanine, valine, leucine, isoleucine, and phenylalanine) that follow a reversible first-order kinetic model of racemization (epimerization) either at low or high temperature. We have estimated the Arrhenius parameters, the activation energy E_a and the frequency factor A, first based on the heating experiments, and later including the fossil data. Valine shows no appreciable differences in E_a and A in both estimations, and could then be used with confidence in dating studies. In a lesser extension this also applies to alanine, phenylalanine, leucine, and glutamic acid. Aspartic acid shows a great difference between the temperature-induced and the time-induced racemization kinetic models, and it should be used with special care in dating studies, since diagenetic racemization in aspartic acid is extremely sensitive to the thermal history of the site.

INTRODUCTION

The role of optical activity in living organisms has long been known [1]. Living organisms contain proteins that are almost exclusively L-amino acids, that start racemizing when the organism dies (i.e., the enzymatic reactions maintaining the disequilibrium reaction cease). Amino acid racemization (epimerization) is well established as a dating method for fossil mollusc shells; it has been applied to fossil bones with generally less success [2–13], and it has been less widely studied in fossil teeth enamel [5] and dentine [14–16]. Extensive

and very successful forensic work has been undertaken using aspartic acid racemization in modern human teeth for determination of age at death [17,18].

Bear fossils are very common in the European paleontological record. Usually, they appear as large monospecific bone and tooth accumulations without clear stratigraphical relationship. In some localities hominid remains are associated with the bear fossil [19].

Amino acid racemization can be modeled reasonably accurately by a reversible first-order kinetic (FOK) model [20] where $k_D/k_L = 1.00$ and D/L is the racemization ratio (Fig. 1a). D-*Allo*-isoleucine/L-isoleucine

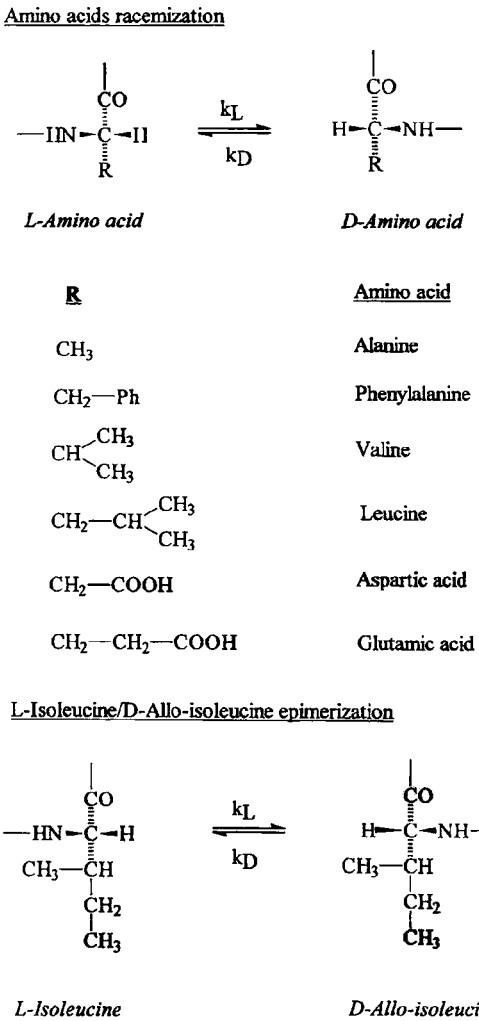

Figure 1 (a) Amino acids racemization reaction for alanine, phenylalanine, valine, leucine, and aspartic and glutamic acids. (b) L-Isoleucine/D-*allo*-isoleucine epimerization reaction.

(A/I) epimerization rate can also be modeled quite accurately by a reversible FOK model where $k_D/k_L = 1.30$ and A/I is the epimerization ratio [21].

To use amino acid D/L ratios for dating or paleotemperature analysis, variations in the rate of racemization (epimerization) with temperature must be known, or, in other words, the Arrhenius parameters E_a (the activation energy) and A (the frequency factor) must be estimated for each amino acid.

The classical way to carry out these estimations is to take samples of dentine of modern bear teeth and heat them at three different temperatures for variable time periods. The estimated k_L value for each amino acid at each temperature can be calculated by a simple linear regression of the FOK transformed D/L (A/I) measurements on time [20]:

For racemization

$$2k_L t = C + \ln \frac{1 + \text{D/L}}{1 - \text{D/L}} \tag{1}$$

For epimerization

$$1.77k_L t = C + \ln \frac{1.30(1 + \text{A/I})}{1.30 - \text{A/I}} \tag{2}$$

The k_L values can be calculated from the slope of the regressions. The basic procedure for estimating the Arrhenius parameters A and E_a thus involved taking three estimated k_L values (and their associated temperatures) for each amino acid and performing a simple linear regression, where ln A is the intercept and $-E_a/R$ is the slope (R is the ideal gas constant, 1.987×10^{-3} kcal mol^{-1} K^{-1}). The major problem of establishing kinetic patterns by this approach of studying modern teeth at high temperatures is that these experimental results may not precisely parallel the patterns that occur at low temperatures over long time periods in fossil material. The "racemization (epimerization) kinetics" must be taken, at least, as the result of two different processes: one reversible (racemization/epimerization) and another irreversible (hydrolysis) affecting proteins and peptides. It is known that fossil material undergo diagenesis that causes changes in the kinetics of racemization (epimerization) over time [22,23]. Most of the racemization takes place at the terminal position of the peptide chains (mainly amino terminal). Over time, progressive hydrolysis occurs and this exposes the slowly racemizing internal amino acids to faster racemizing terminal positions, so that racemization continues but at a rate constrained by the rate of hydrolysis. Thus, it is not clear whether these various processes (hydrolysis and racemization) follow exactly the same pattern under both high and low temperature conditions, differing only in rate, and thus, it

is also uncertain whether the kinetic patterns observed in heated modern bear teeth dentine can be used to accurately model the kinetic behavior of fossil teeth. The problem of extrapolation of kinetics from high to low temperature situations can be avoided by establishing kinetic patterns directly from a series of dated fossil materials, but this approach also presents problems: it is difficult to find a time series of known-age teeth, and it is nearly impossible to find any time series of known-age teeth preserved at different temperature conditions, in order to estimate Arrhenius parameters. In this paper, we have followed the same approach of Goodfriend and Meyer [24] on *Trochoidea seetzeni* shells to estimate Arrhenius parameters, that is, we have heated modern bear teeth dentine samples at three different temperatures over variable time periods, and we have obtained the E_a and A values for seven amino acids (aspartic and glutamic acids, alanine, valine, leucine, isoleucine, and phenylalanine). Later, we have made a second estimation of these Arrhenius parameters for these seven amino acids but including as a fourth point in the regression a fossil series of known-age (dated by independent methods) and known temperature preservation conditions, and we have compared the variations in the E_a and A values.

EXPERIMENTAL

The general plan of the study involved sealing sets of bear teeth powder into a series of N_2-filled tubes with moisture present, placing the tubes under constant temperature conditions, and periodically removing a tube for analysis of D/L-amino acid ratios.

The glassware used for the analyses (except the Pasteur pipettes) was cleaned by baking in an oven at 500°C for about 2 h. Eppendorf plastic micro test tubes, plastic micropipette tips, and Pasteur pipettes were new from the factory. Teflon liners and septa were thoroughly washed with petroleum ether and acetone and rinsed three times with ultraclean water. All the water used in the analysis was Milli-Q quality from Millipore. Concentrated hydrofluoric and hydrochloric acids and trifluoroacetic acid anhydride were Merck analytical grade. Thionyl chloride was purchased from Fluka AG. Isopropyl alcohol and *n*-hexane were Merck HPLC grade, and dichloromethane was Merck spectroscopy grade.

Teeth Samples

The bear teeth fossil samples that we have used in our study are as follows:

1. A relatively recent bear tooth sample that appeared close to some bear bones in the Eirós (Galicia) cave. A bone sample was ^{14}C (AMS) dated [25] resulting in an estimated age of 24,090 ± 440 yr BP.
2. A bear tooth of intermediate age that appeared in La Lucía (Asturias) cave. In the bear remains bearing bed from La Lucía cave some small stalactites appeared, and a thin flowstone 2–3 cm thick sealed the bone and teeth bearing bed. Two calcite samples were U/Th-dated and the estimated age was 76,400 ± 2700 yr BP [19].
3. A bear tooth sample of older age that appeared in the Sima de los Huesos (Atapuerca, Burgos). A combination of electron spin resonance (ESR) and U-series dating methods on the remains of the Sima de los Huesos bear gave a probable date of 320,000 ± 4000 yr BP [26].

For the kinetics analysis of modern bear teeth dentine we have employed *Ursus americanus* teeth from a skull that was purchased in 1986, which is exposed in the Historical and Mining Museum of the Madrid School of Mines.

Sample Preparation

Because crushing was not possible, powder samples from canines and lower molar roots were obtained with an odontologist radial milling cutter; a slight heating was unavoidable. We avoided sampling the pulp cavity where dry soft tissues still remained. Samples of 50 mg of powdered dentine were obtained from the innermost part of the crown via drilling the tooth with a dental diamond drill. Powder from the outer part of the root, up to the limit of 1 mm deep, was rejected. Cementum layers were never sampled. This process produced an unavoidable slight sample heating. We drilled near the crown/root boundary avoiding black-colored root zones. Tooth powder was stored in small plastic boxes new from the factory.

Preparation of Tubes

Approximately 100 mg of tooth powder and 2 g of quartz sand (deeply precleaned by oven baking at 600°C for 6 h) were placed into borosilicate glass test tubes (135 mm long and 13 mm wide) with screw caps and Teflon septa. 120 μL of ultraclean water was added via syringe. The top of the tube was fitted into rubber tubing connected to a vacuum-N_2 line, and the tube was alternately exposed to vacuum and to N_2, repeated three to four times to flush out all the air; while under

nitrogen, the screw cap of the tube was tightly closed. A blank was prepared to check for spurious amino acid contamination.

Kinetic Experimental Conditions

The tubes were placed in a rack and put into an oven. The samples were kept at 65°C, 85°C, and 105°C (measured by a type K thermocouple) for up to 53 days for the 65°C and 85°C experiments, and for up to 71 days for the 105°C experiment.

Sampling

A tube of each temperature experiment was removed at the intervals as shown in Tables I–III. The tubes were opened and dried. The teeth powder in each tube was separated from the sand by sieving, and it was washed with distilled water, sonicated, and dried under vacuum.

Dialysis

Before performing hydrolysis and amino acid derivatization, the samples were dialyzed to eliminate free amino acids and other small molecules. With this process, we aimed to remove foreign amino acids and to obtain a homogeneous size protein (from collagen) molecule. We have employed a modification of the method proposed by Marzin [27]. The powder sample, approximately 50 mg of dentine, was dissolved in 1 mL of 2 N hydrochloric acid and sonicated. After the addition of 5 mL PBS buffer, the sample was dialyzed at 3500 Da (Spectra/Por mnco 3500 membrane) with magnetic stirring at room temperature during a 20-h period using the buffered solution.

Analysis of D/L Ratios

The D/L ratios of the seven amino acids were measured by gas chromatography of the *N*-trifluoroacetyl isopropyl ester derivatives using a Chirasil-Val column. The samples were prepared according to Goodfriend's [24] method:

Hydrolysis was carried out in a mixture of 12 N hydrochloric acid (2.9 μL/mg of sample) and 6 N hydrochloric acid (100 μL), in test tubes with Teflon-lined screw caps closed under a nitrogen atmosphere, in a heating block at 100°C for 20 h. Samples were transferred to conical 1.5 mL Eppendorf plastic micro test tubes with caps, concentrated hydrofluoric acid (1.25 μL/mg of sample) was added, and the tubes were mixed with a mechanical Vortex shaker, and centrifuged for 4 min in an Eppendorf centrifuge. The supernatant was transferred into new 1.5 mL Eppendorf micro test tubes, frozen in liquid nitrogen, and vacuum-dried in a plastic desiccator. Samples were redissolved with 80 μL of ultraclean water, mixed in the Vortex shaker, centrifuged for a few seconds to get all droplets down, and transferred into 2 mL glass vials, with screw caps and Teflon lined septa. Water was evaporated from the cap-covered vials, which were not tightly closed, in the plastic desiccator under vacuum conditions.

The first step in amino acid derivatization was esterification with 250 μL of 3 M thionyl chloride in isopropanol. The vials were tightly closed under nitrogen and allowed to react on the heating block at 100°C for just 1 h. Afterwards, the vials were opened, but not uncovered, in a hood, and were vacuum-dried in a plastic desiccator, this process being performed to the point of dryness, but not longer. The second step in derivatization was *N*-trifluoroacetylation with 150 μL of trifluoroacetic acid anhydride (25% in dichloromethane). The vials were tightly closed under nitrogen and heated at 100°C for 5 min on the heating block. They were then

Table I Amino Acid D/L Ratios[a] of Experimental Samples Heated at 65°C

Time (h)	Asp	Glu	Ala	Phe	Leu	*Allo*/Ile	Val
24	0.052	0.008	0.010	0.010		0.005	0.004
36	0.063	0.013	0.011	0.011	0.009		0.004
48	0.063	0.012	0.010	0.009	0.009	0.005	0.004
108	0.088	0.012	0.011		0.009		0.004
240	0.184		0.013	0.013	0.011		0.005
432	0.165		0.009		0.008		0.005
672	0.266	0.016	0.014		0.011		0.006
864	0.267		0.015	0.015	0.011	0.011	0.005
936	0.348	0.019	0.016	0.014	0.011	0.008	0.006
1272	0.333	0.017	0.016	0.013			0.005

[a]The uncertainty associated with these measurements is ±3%.

Table II Amino Acid D/L ratios[a] of Experimental Samples Heated at 85°C

Time (h)	Asp	Glu	Ala	Phe	Leu	*Allo*/Ile	Val
24	0.150	0.011	0.012	0.010	0.007		0.004
36	0.204	0.010					
48	0.248	0.018	0.016	0.017	0.014		0.005
108	0.223		0.012		0.009		0.005
240	0.557	0.023	0.021	0.018	0.013	0.006	0.007
432	0.574	0.025	0.019	0.020	0.012		0.007
672	0.867	0.068	0.036	0.035	0.017	0.018	0.014
864	0.817	0.060	0.040	0.039	0.021	0.018	0.014
936	0.839	0.058	0.038	0.038	0.019	0.016	0.014
1272	0.847	0.073	0.048	0.045	0.024	0.023	0.021

[a]The uncertainty associated with these measurements is ±3%.

allowed to cool, and subsequently opened in a hood, in which the dichloromethane solvent and the unreacted trifluoroacetic acid anhydride were evaporated under a gentle flow of nitrogen. Later the samples were dissolved in 125 μL of *n*-hexane, shaken in the Vortex, and the solvent evaporated in a stream of nitrogen to a final volume of 15–25 μL and transferred to 150 μL injection vials.

A 0.2-μL sample was injected into a Hewlett-Packard 5890 gas chromatograph. The injection port was kept at 215°C and set for splitless mode for the first 75 s, at the beginning of which the sample was injected and later set to split mode. We used helium as the carrier gas, at a column head pressure of 40 kPa, and a Chirasil-Val fused silica column (25 m × 0.25 mm × 0.12 μm) from Chrompack. The gradients used were as follows: 50°C (1 min), heating at 40°C/min to 115°C, 12 min at 115°C, heating at 3°C/min to 190°C, 10 min at 190°C, cooling to 50°C, and maintenance at this temperature between runs (or at 80°C if the time

Table III Amino Acid D/L Ratios[a] of Experimental Samples Heated at 105°C

Time (h)	Asp	Glu	Ala	Phe	Leu	*Allo*/Ile	Val
0	0.050						
24	0.240	0.020					
24	0.270	0.020	0.011	0.011	0.012		0.011
36	0.670	0.030	0.016	0.020	0.016		0.005
48	0.280	0.020		0.012	0.010		
60	0.780	0.070	0.024	0.022	0.017	0.012	0.009
72	0.840	0.060	0.115		0.023		
108	0.930	0.060	0.034	0.039	0.023	0.018	0.016
168	0.960	0.100	0.029	0.036	0.020	0.020	0.014
204	0.910	0.120	0.130		0.044		
240		0.160	0.070	0.098	0.031	0.039	0.042
276		0.160	0.058	0.065	0.055	0.040	0.033
348		0.210	0.066	0.073	0.039	0.042	0.041
432		0.240	0.085	0.090	0.043	0.064	0.050
504	0.990	0.270	0.096	0.101	0.047	0.067	0.062
576	0.990	0.340		0.136			
672	0.950	0.130	0.053	0.054	0.054	0.036	0.033
744		0.360	0.169	0.135	0.076	0.135	0.109
864		0.420	0.212	0.204	0.188	0.167	0.077
936	0.920	0.480	0.252	0.262	0.126	0.174	0.146
1080	0.880	0.440	0.259	0.272	0.131	0.210	0.158
1272	0.860	0.570	0.462	0.374	0.198	0.323	0.234
1440	0.750	0.540	0.412	0.355	0.199	0.233	0.229
1704	0.920	0.640	0.608	0.556	0.300	0.410	0.316

[a]The uncertainty associated with these measurements is ±3%.

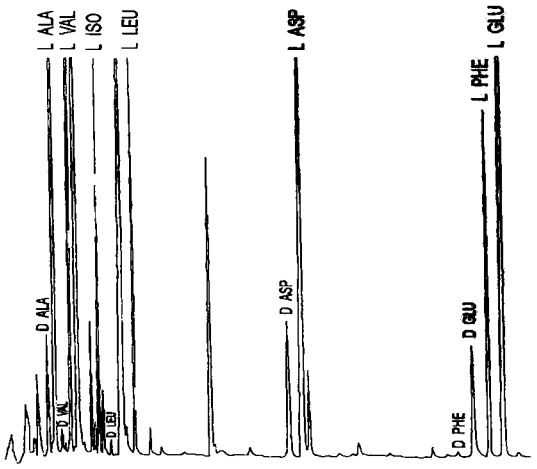

Figure 2 Typical chromatogram of the tooth of a fossil bear with the identification of the D- and L-amino acid peaks.

between runs was longer, typically overnight). The detector was a nitrogen–phosphorus detector set at 300°C. Integration of the peak areas was carried out using the HP Peak96 integration program from Hewlett-Packard, which runs on a PC computer. The sensitivity limits of the method could be fixed according to the induced racemization method (0.00–0.03 depending on the amino acid considered) and the minimum amino acid concentration detectable in the samples. As a laboratory routine D/L-valine, D/L-alanine, D/L-leucine, D-*allo*-isoleucine/L-isoleucine, D/L-aspartic acid, D/L-phenylalanine, and D/L-glutamic acid peaks were identified. Figure 2 shows a typical chromatogram with the identification of the D- and L-amino acid peaks.

Estimation of Arrhenius Parameters

The Arrhenius parameters, E_a (the activation energy) and A (the frequency factor), which describe the relation of racemization (epimerization) rates to temperature, were estimated for the teeth samples based on reversible first-order kinetic rate constants (k_L) determined from heating experiments run at three different temperatures. Besides the initial ($t = 0$) D/L value, D/L ratios of samples heated up to 53 days for the 65°C and 85°C experiments, and up to 71 days for the 105°C experiment were used in the calculations (Tables I–III). However, for aspartic acid, only the initial D/L value and the D/L values up to 576 h for the 105°C experiment were used, owing to the strong nonlinearity of D/L with time in this amino acid at this high temperature. For estimation of k_L values, the D/L ratios were converted to their FOK transforms. For D/L enantiomer ratios, the transformation was done by Eq. (1). For A/I the transformation was done by Eq. (2).

The estimated k_L value for each amino acid for each temperature was calculated by a simple linear regression of the FOK-transformed D/L measurements on time (in hours). The k_L values (Table IV) were calculated from the slope of the regression by the following equations:

For racemization

$$k_L = 0.5 \times \text{slope} \tag{3}$$

For isoleucine epimerization

$$k_L = 0.565 \times \text{slope} \tag{4}$$

After Arrhenius equation, $\ln k_L$ is inversely related to the temperature, according to the following general equation:

$$\ln k_L = \ln A - \frac{E_a}{R}\frac{1}{T} \tag{5}$$

where $\ln A$ is the intercept, $-E_a/R$ is the slope, and T is the temperature (in K). The activation energy E_a can be calculated from the slope by the following equation:

$$E_a = -\text{slope} \times R \tag{6}$$

where R is the gas constant ($R = 1.987 \times 10^{-3}$ kcal mol^{-1} K^{-1}). The basic procedure for estimating the

Table IV Estimated k_L Values of Samples from Heating Experiments, Used in the Estimation of Arrhenius Parameters

	k_L (h^{-1})[a]						
Temp. (°C)	Asp	Glu	Ala	Phe	Leu	*Allo*/Ile	Val
65	2.5×10^{-4}	5.0×10^{-6}	5.0×10^{-6}	3.0×10^{-6}	2.5×10^{-6}	5.1×10^{-6}	1.0×10^{-6}
85	1.0×10^{-3}	5.0×10^{-5}	3.0×10^{-5}	3.0×10^{-5}	1.0×10^{-5}	1.7×10^{-5}	1.0×10^{-5}
105	4.2×10^{-3}	4.5×10^{-4}	3.5×10^{-4}	3.0×10^{-4}	1.5×10^{-4}	2.3×10^{-4}	2.0×10^{-4}

[a] k_L values were determined at 65°C (1272 h), 85°C (1272 h), and 105°C (1704 h), except for aspartic acid at 105°C (576 h) to discard the inversion of racemization.

Table V Estimates of Arrhenius Parameters, E_a and A from the Heating Experiments on Modern Bear Teeth

Amino Acid	E_a (kcal mol^{-1})	ln A (h^{-1})
Ala	26.9	28.3
Allo/Ile	23.9	23.7
Asp	17.9	19.0
Glu	28.5	31.0
Phe	29.2	31.4
Val	33.5	36.6
Leu	25.8	26.0

Arrhenius parameters A and E_a thus involved taking the three estimated k_L values (and their three associated temperatures) for each amino acid and performing a simple linear regression, which gives A and the slope, from which E_a is calculated (Table V).

The k_L values estimated from the heating experiments on modern bear teeth (and their three associated temperatures) were combined with the k_L value calculated from a series of three radio-dated fossil samples (assuming a current mean annual temperature of 12.5°C in the caves over time and changing the y^{-1} unit to h^{-1} unit, Tables VI and VII), and a simple linear regression was performed in order to obtain a new set of A and E_a values which included the fossil samples (Table VIII).

RESULTS AND DISCUSSION

Heating Experiments in Modern Bear Teeth Dentine

Table I shows the D/L (A/I) racemization (epimerization) ratios for the seven amino acids under study on heating at 65°C up to 1272 h (53 days). As expected, aspartic acid was the fastest racemizing amino acid attaining a high D/L ratio of 0.33 at 1272 h; Table IV shows the k_L rates calculated from the D/L (A/I) ratios in Table I. The R^2 linear regression coefficients, the Student t test, and the probability P for $(n - 2)$ freedom degrees and a two-queues proof are in Table IX. Aspartic acid has a k_L that is nearly two orders of magnitude larger than the k_L values of the other amino acids. There is a group of three amino acids with k_L values around 5.0×10^{-6} h^{-1} (isoleucine, glutamic acid, and alanine), two amino acids show values around 3.0×10^{-6} h^{-1} (phenylalanine and leucine), and the slowest racemizing amino acid is valine ($k_L = 1.0 \times 10^{-6}$ h^{-1}).

Table II summarizes the D/L (A/I) racemization (epimerization) ratios for the same seven amino acids on heating at 85°C up to 1272 h (53 days). Again aspartic acid reaches a high D/L ratio of 0.85. The k_L values calculated from these D/L (A/I) ratios appear in Table IV. The R^2 linear regression coefficients, the Student t test, and the probability P for $(n - 2)$ freedom degrees and a two-queues proof are in Table IX; as a general observation, the R^2, t, and P values are better for this series. However, the k_L values at 85°C do not group in the same way as at the lower temperature; of course, aspartic acid shows again a k_L value nearly two orders of magnitude larger than the other amino acids (1.0×10^{-3} h^{-1}), and its k_L is exactly four times the k_L value at 65°C. Glutamic acid shows as the second fastest racemizing amino acid at 85°C, alanine and phenylalanine grouped together with k_L values of 3.0×10^{-5} h^{-1}, and leucine and valine show the slowest racemization rates (1.0×10^{-5} h^{-1}). The A/I epimerization rate is also slow at this temperature (1.7×10^{-5} h^{-1}).

Table III shows the D/L (A/I) racemization (epimerization) ratios for the seven amino acids under study on heating at 105°C up to a longer time period of 1704 h (71 days). At this temperature, a curious phenomenon occurs in the D/L racemization ratios of aspartic acid: after 576 h of heating, the D/L ratio reaches the equilibrium value of 0.99, but with longer heating times (from 672 h to 1440 h) the D/L ratio decreases to 0.75, raising again to 0.92 at 1704 h (Fig. 3a). This fact is not new in amino acid racemization studies: some authors [28,29] describe an "apparent racemization kinetic reversal" for aspartic acid in the peptide fraction of molecular weight >1000 Da of some bivalve shells (*Ostrea sp.*). Figure 3b shows D/L glutamic acid racemization ratios against time (h) at 105°C for comparison with aspartic acid. As we have written before, the racemization kinetics must be taken, at least, as the result of two different processes: one reversible, racemization, and another irreversible, hydrolysis affecting

Table VI Amino Acid D/L Ratios[a] of Fossil Samples Dated by Independent Methods

Dating	Time (yr)	Asp	Glu	Ala	Phe	Leu	*Allo*/Ile	Val
^{14}C	24,090 ± 440	0.070	0.033	0.014	0.265	0.048	–	0.015
U/Th	76,424 ± 2700	0.130	0.064	0.062	0.170	0.030	–	0.044
U-ser/ESR	320,000 ± 4000	0.320	0.115	0.264	0.302	0.138	–	0.106

[a]The uncertainty associated with these measurements is ±3%.

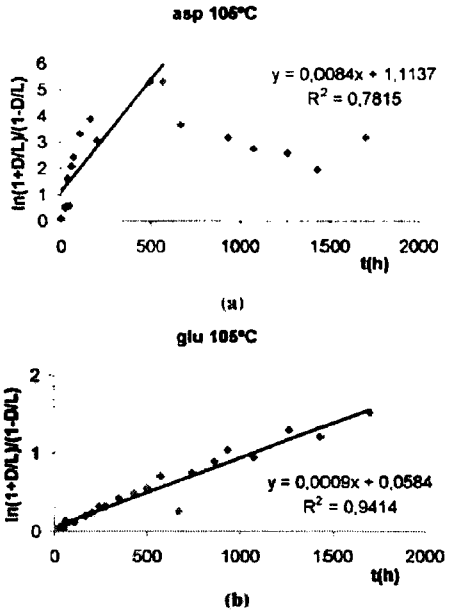

Figure 3 (a) D/L-Aspartic acid racemization ratios against time (h) showing the "apparent kinetic reversal" at 105°C. (b) D/L-Glutamic acid racemization ratios against time (h) at 105°C for comparison.

proteins and peptides. The existence of a certain hydrolysis resistance in the peptide bonds where hydrophobic amino acids are linked to aspartic acid has been reported [28,29], and since most of the racemization takes place at the terminal positions of the peptide chains, an "apparent racemization reversal" can be observed when internal, less racemized aspartic acid is released.

Table IV summarizes the k_L kinetic rates for the seven amino acids on heating at 105°C. The R^2 linear regression coefficients, the Student t test, and the probability P for $(n - 2)$ freedom degrees and a two-queues proof are in Table IX; these values are on the whole even better than the values of the series at 85°C.

Table VII Estimated k_L Values of Fossil Samples, Used in the Estimation of Arrhenius Parameters

	k_L (yr^{-1}) (at 12.5°C[a])
Asp	1.0×10^{-6}
Glu	2.5×10^{-7}
Ala	1.0×10^{-6}
Phe	2.5×10^{-7}
Leu	3.5×10^{-7}
Allo/Ile	–
Val	3.0×10^{-7}

[a]Current mean annual temperature (CMAT) estimated in caves for the fossil samples.

Table VIII Estimates of Arrhenius Parameters, E_a and A as Table V, but Including the Fossil Samples Series as an Additional Point in the Estimates Based on the Heating Experiments on Modern Bear Teeth

Amino Acid	E_a (kcal mol)	ln A (h^{-1})	ΔE_a[a]
Ala	34.8	39.5	7.9
Asp	42.0	52.7	24.1
Glu	39.1	45.7	10.6
Phe	38.0	43.7	8.8
Val	35.9	39.9	2.4
Leu	35.1	39.0	9.3

[a]ΔE_a is the positive difference between these E_a values and those in Table V.

Again, aspartic acid is the fastest racemizing amino acid, with a k_L nearly an order of magnitude larger than the k_L of glutamic acid, the second faster racemizing amino acid. As at 85°C, alanine and phenylalanine grouped together with k_L values around 3.0×10^{-4} h^{-1}, and the slowest racemizing amino acids are valine and leucine, with k_L values of 2.0×10^{-4} and 1.5×10^{-4} h^{-1} respectively. The k_L value for the epimerization of L-isoleucine is also low (2.3×10^{-4} h^{-1}) following thus the same trend as at 85°C.

From the observation of the k_L values in Table IV and the previous discussion, we could observe a relationship between the racemization (epimerization) rates of amino acids and the chemical structure of the R groups of the side chain of these amino acids (see Fig. 1a). The faster racemizing amino acids (aspartic and glutamic acids, in this order) are those amino acids with the most hydrophylic R groups: in fact, the R = CH_2—COOH group on aspartic acid is more hydrophylic than the R = CH_2—CH_2—COOH group on glutamic acid, which has an additional methylene group; aspartic acid is the fastest racemizing amino acid followed by glutamic acid. Moreover, those peptide bonds where aspartic acid is linked to hydrophobic amino acids are the most resistant to the hydrolysis + racemization process, which explains its "apparent kinetic reversal" [29]. Following a decreasing order of k_L values, alanine and phenylalanine form a second group of amino acids, reflecting a similar hydrophobic character for their pendant R groups, methyl and benzyl. Clearly, when the hydrophobic character of the pendant R groups is higher, valine (R = *i*-Pr), leucine (R = *i*-Bu), and isolucine (R = *sec*-Bu), the racemization (epimerization) rates decrease accordingly. Also associated with the relationship between the racemization rate and the more or less hydrophobic chemical structure of amino acids is the influence of moisture on the kinetics of racemization. Some years ago, we started a heating experiment

Table IX Statistical Parameters for the Values Estimated in Tables I–VIII

	Asp	Glu	Ala	Phe	Leu	*Allo*/Ile	Val
65°C							
n	10	7	10	7	8	4	10
R^2	0.9155	0.7231	0.7187	0.5856	0.4935	0.8270	0.5073
Student *t*	9.31	3.61	4.52	2.66	2.42	3.09	2.87
P	0.07	0.17	0.14	0.23	0.25	0.20	0.21
85°C							
n	10	9	9	8	9	5	9
R^2	0.9113	0.8800	0.9492	0.9413	0.8626	0.8703	0.9512
Student *t*	9.07	7.16	11.44	9.81	6.63	4.49	11.68
P	0.07	0.09	0.06	0.06	0.10	0.14	0.05
105°C							
n	12	23	20	20	21	16	18
R^2	0.7815	0.9414	0.8534	0.8990	0.8999	0.9016	0.9147
Student *t*	5.98	18.37	10.24	12.66	13.07	11.33	13.10
P	0.11	0.03	0.06	0.05	0.05	0.06	0.05
Fossil							
n	3	3	3	3	3		3
R^2	0.9968	0.9560	0.9999	0.3566	0.9001		0.9778
Student *t*	17.65	4.66	49.99	0.74	3.00		6.64
P	0.04	0.13	0.01	0.59	0.20		0.10
E_a							
n	3	3	3	3	3	3	3
R^2	0.9982	0.9996	0.9851	0.9990	0.9539	0.9434	0.9885
Student *t*	23.55	49.99	8.13	31.61	4.55	4.08	9.27
P	0.03	0.01	0.08	0.02	0.14	0.15	0.07
E_a fossil							
n	3	3	3	3	3		3
R^2	0.9582	0.9904	0.9921	0.9929	0.9878		0.9982
Student *t*	4.79	10.16	11.21	11.83	9.00		23.55
P	0.13	0.06	0.06	0.05	0.07		0.03

on modern shells and modern bear dentine without adding water to the samples to be heated, and we did not obtain appreciable racemization (epimerization) ratios after many days of heating (T. Torres, P. García-Alonso, L. Canoira, J. F. Llamas, unpublished results, 1996).

The estimation of Arrhenius parameters, E_a and A, has been accomplished in the classical way as is explained in the experimental part and summarized in Table V. The R^2 linear regression coefficients, the Student t test, and the probability P for $(n - 2)$ freedom degrees and a two-queues proof are in Table IX. The activation energy values range from 17.9 kcal mol^{-1} for aspartic acid to 33.5 kcal mol^{-1} for valine; the fastest racemizing amino acid shows the lower activation energy, and one of the slower racemizing amino acids, valine, shows the higher activation energy, but excluding these extreme values, there is no clear correlation between racemization (epimerization) rate and activation energy. On comparing these activation energies with those obtained in similar work [24] carried out heating modern shells of *Trochoidea seetzeni*, we also observed a variability of results: from practically negligible differences for alanine and glutamic acid (+0.26 and +0.47 kcal mol^{-1}, respectively; the + sign means a higher value in our work, and the − sign the opposite) to very different values for aspartic acid (−12.00 kcal mol^{-1}). The differences in activation energy for the remaining amino acids are −1.70 kcal mol^{-1} for D-*allo*-isoleucine/L-isoleucine and +2.92 kcal mol^{-1} for phenylalanine (valine and leucine were not considered in the shell work). The very big difference observed for aspartic acid (in fact, the only meaningful one) probably reflects the very different contents and situation of aspartic acid units in the dentine of bear teeth and in the intracrystalline proteins of *Trochoidea*

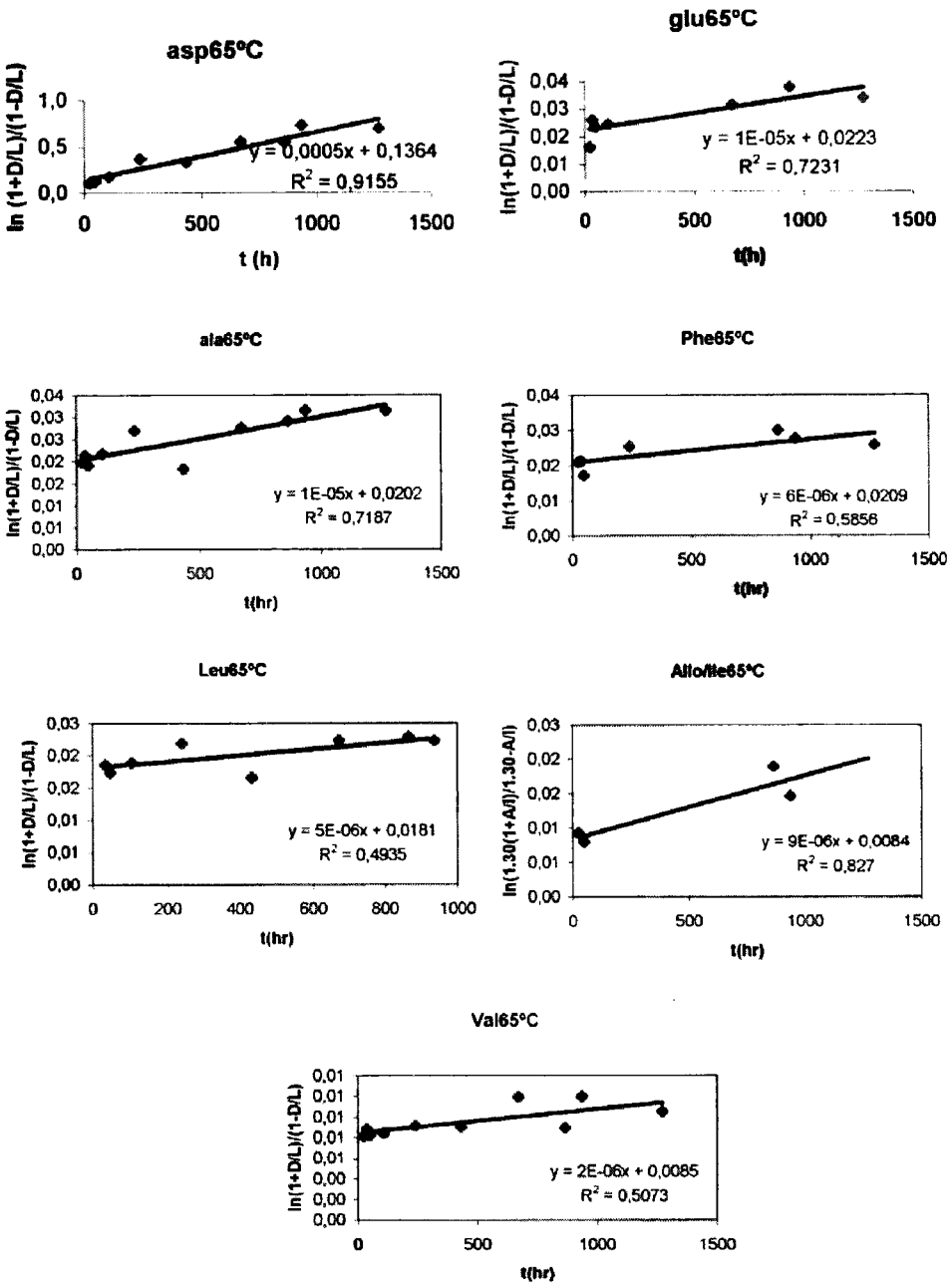

Figure 4 Amino acid D/L (A/I) ratios against time (h) of experimental samples heated at 65°C.

shells. Moreover, the activation energies obtained for the amino acids under study are in the range currently estimated for biochemical reactions [20] (20–40 kcal mol^{-1}). The very low activation energy value obtained for aspartic acid means that the racemization reaction for this amino acid is not very sensitive to the effect of the temperature, and the opposite for valine and leucine [20].

As to the frequency factor A (tabulated as the ln A in Table V), aspartic acid shows also the smaller value (19.0) and valine the bigger value (36.6). Glutamic acid, phenylalanine, and alanine show values around 30, while leucine and isoleucine show very similar values around 25. All these values without exception are higher than those found in the heating experiment of *Trochoidea* shells [24].

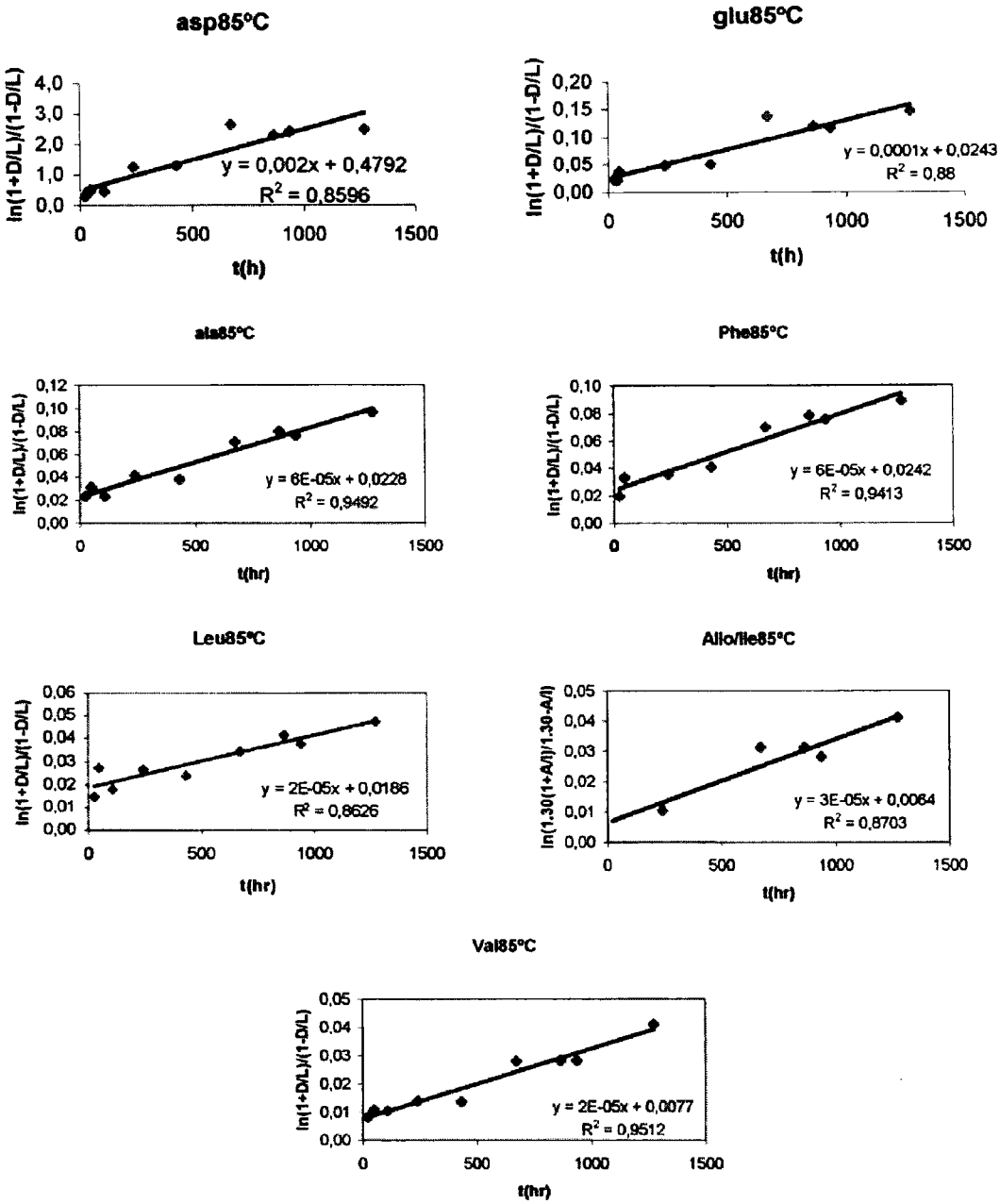

Figure 5 Amino acid D/L (A/I) ratios against time (h) of experimental samples heated at 85°C.

Comparison of Kinetics in Modern and Fossil Bear Teeth Dentine

To compare the racemization kinetics induced by the heating in modern bear teeth dentine with the racemization kinetics induced by the time in fossil bear teeth dentine, we have carried out the study of three fossil bear dentine samples whose localities have been dated by independent methods (see the Experimental part, section *Teeth Samples*).

Table VI summarizes the D/L ratios of the amino acids under study for this fossil series, except isoleucine whose A/I data could not be obtained from the chromatograms on Chirasil-Val column because A/I peaks

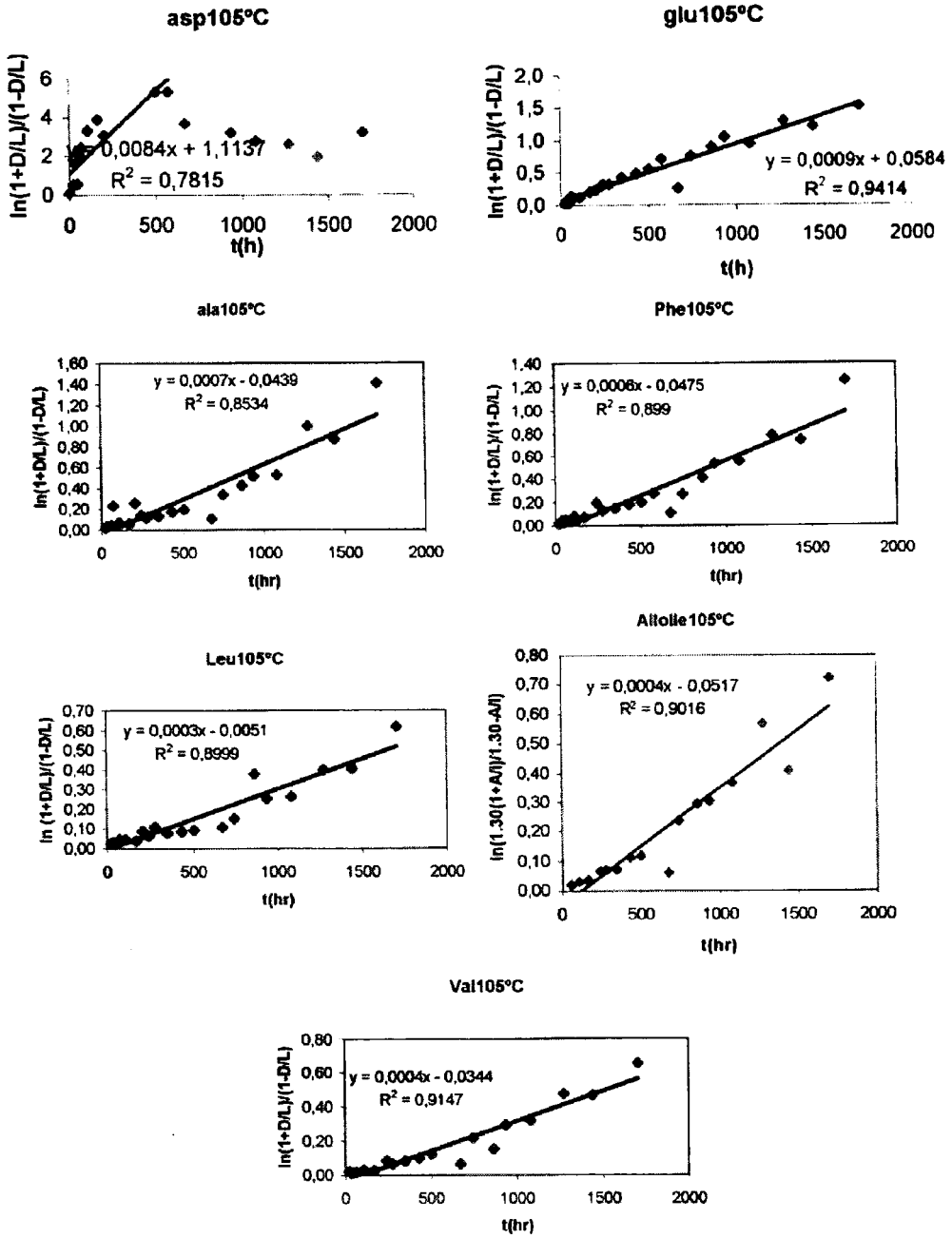

Figure 6 Amino acid D/L (A/I) ratios against time (h) of experimental samples heated at 105°C.

overlap with other amino acid or junk peaks; thus the discussion for this amino acid is excluded also in the text that follows. Table VII shows the k_L values for the fossil samples estimated from the D/L data in Table VI using Eq. (1). The R^2 linear regression coefficients, the Student t test, and the probability P for $(n-2)$ freedom degrees and a two-queues proof are in Table IX; the values for phenylalanine have no statistical meaning, but they have been included for the sake of completeness. For the fossil samples, the racemization rate follows a different trend when compared with the heated samples. Again, aspartic acid is the fastest racemizing amino acid (1.0×10^{-6} y^{-1}), but with the same rate as alanine; leucine, valine, phenylalanine,

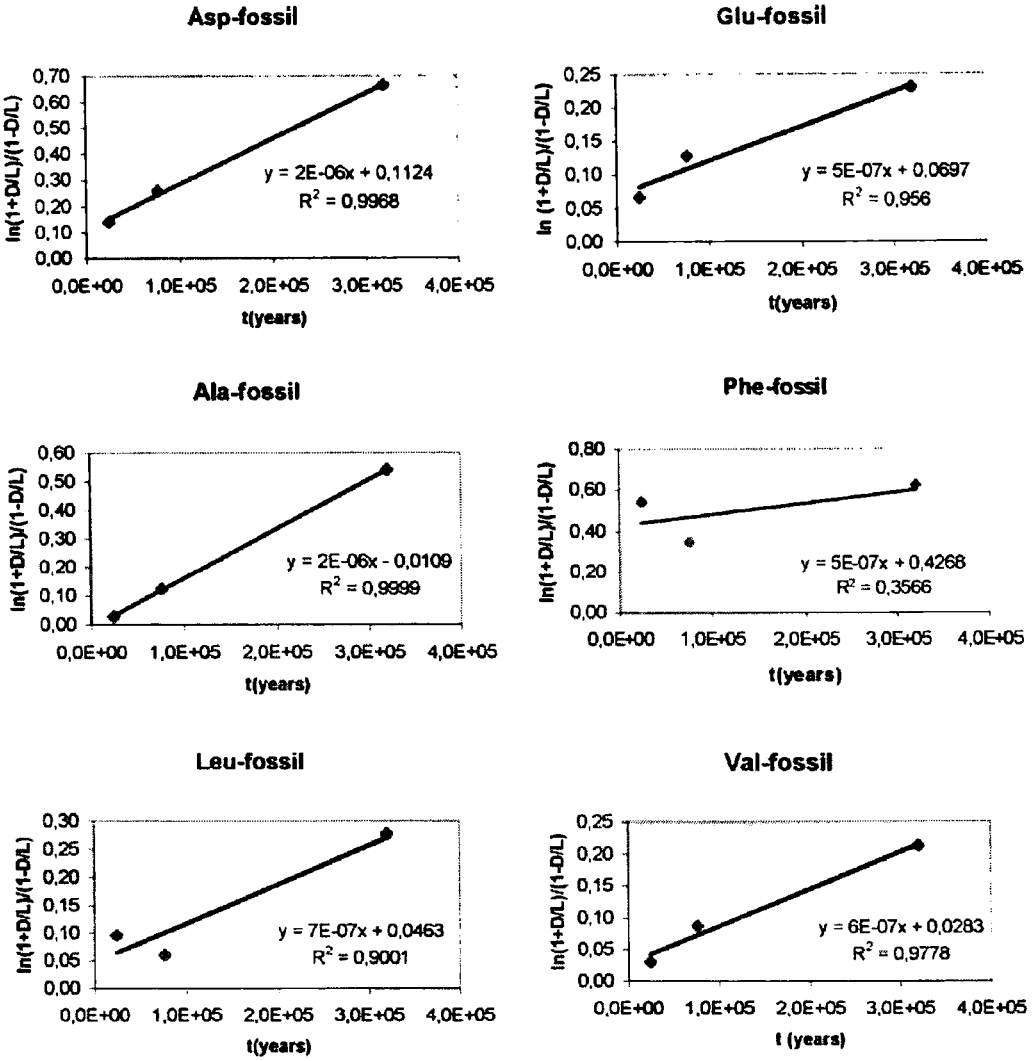

Figure 7 Amino acid D/L ratios against time (y) of fossil samples dated by independent methods.

and surprisingly, glutamic acid showed slower racemization rates, with k_L values of 3.5, 3.0, 2.5, and 2.5 $\times$ 10^{-7} y^{-1}, respectively (the k_L value for phenylalanine should be taken as doubtful, owing to its high P value).

We proceeded later to a reestimation of the Arrhenius parameters, E_a and A, but including the k_L values of the fossil series from Table VII. The most important decision in doing this reestimation was to decide the preservation temperature of the fossils. Caves are ideal environments because perennial, saturated moisture is present in the fossil-bearing muddy sediments and a very stable thermal history can be expected. In Spanish caves, the air temperature change during an annual cycle is less than 0.5°C [25], and would therefore be negligible inside the fossil-bearing sediment. Thus, we have estimated for our fossil bear teeth a current mean annual temperature (CMAT) of 12.5°C, and we have used this value in the reestimation of the Arrhenius parameters. Table VIII shows the reestimates of the Arrhenius parameters including the fossil samples series as an additional point in the estimates based on heating experiments on modern bear teeth. The estimations show very good statistical values except aspartic acid. The last column of this Table VIII, ΔE_a, summarizes the always positive differences between these activation energies and those on Table V, from which some interesting conclusions could be inferred.

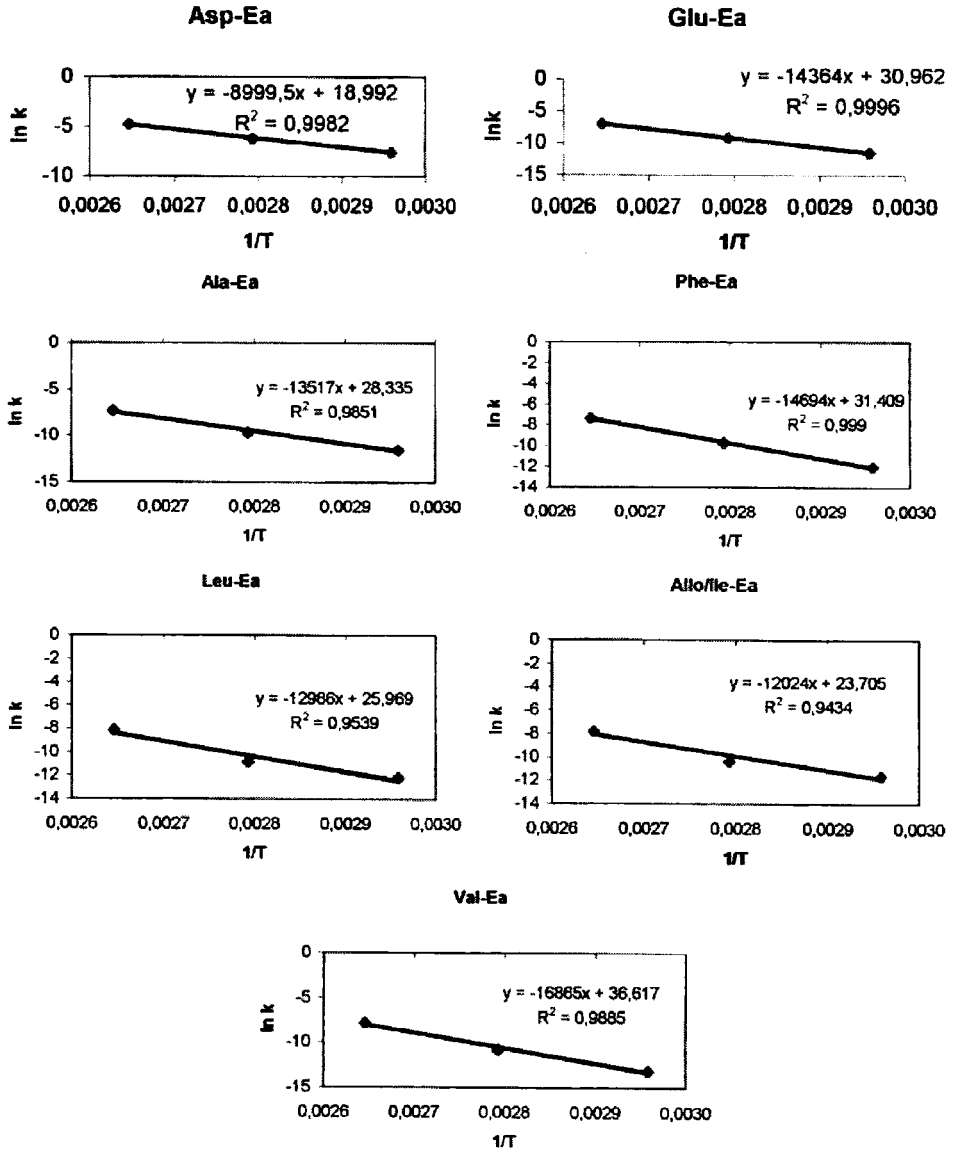

Figure 8 Ln k_L values of samples from heating experiments against $1/T$ (K^{-1}) used in the estimation of Arrhenius parameters in Table V.

CONCLUSIONS

The seven studied amino acids (aspartic and glutamic acids, alanine, valine, leucine, isoleucine, and phenylalanine) follow a reversible first-order kinetic model of racemization (epimerization) either at low (12.5°C) or high temperature (65, 85, and 105°C).

The racemization rates of these seven amino acids, measured by their k_L kinetic constants, are somewhat related to the chemical structure of the R side chain groups. The amino acids with the most hydrophilic R groups (aspartic and glutamic acids) racemize faster, and the amino acids with the most hydrophobic R groups racemize more slowly.

The Arrhenius parameters, the activation energy E_a and the frequency factor A, have been estimated for these seven amino acids, first based on the heating experiments data and later including the fossil data. The higher values of E_a mean that the influence of the temperature on the racemization kinetics is in general bigger when the fossil data are included.

The amino acid with the highest E_a in the heating experiments, valine, only increases its E_a value by 2.4 kcal mol^{-1} on including the fossil data. This fact

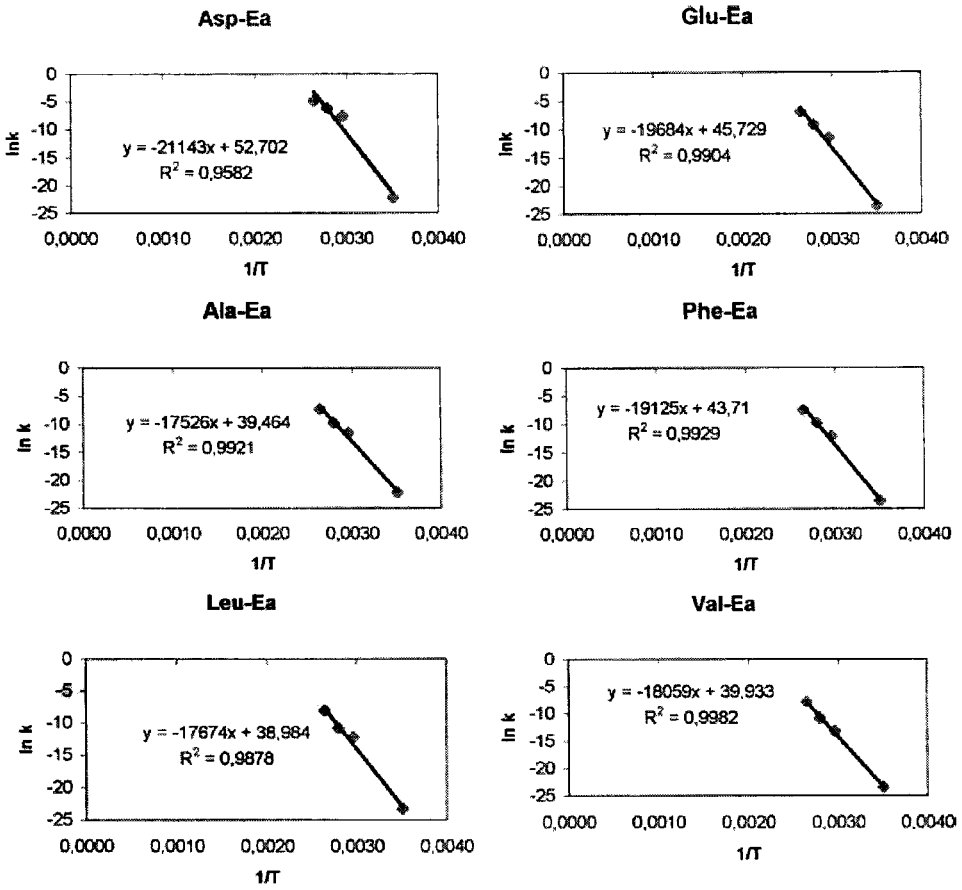

Figure 9 Ln k_L values of fossil samples and samples from heating experiments against $1/T$ (K^{-1}) used in the estimation of Arrhenius parameters in Table VIII.

could suggest that when the E_a is high, the temperature-induced racemization mechanism is very similar to the time-induced racemization mechanism. Moreover, since this amino acid shows a slow racemization rate, it could be used with some accuracy to date fossil teeth of older ages.

The amino acid with the lower E_a in the heating experiments, aspartic acid, showed the higher E_a when including the fossil data (ΔE_a = 24.0 kcal mol^{-1}, 10-fold the ΔE_a for valine). This fact could suggest that in amino acids with low E_a, the temperature-induced and the time-induced racemization mechanisms are very different, and special care should be taken when using this amino acid for dating purposes in fossils of older ages. In particular, the kinetic model obtained in the heating experiments should be avoided in dating studies, and a time series of teeth samples of well-known age should be used to establish the regression model D/L ratio vs. time. The amino acids where E_a on heating experiments range from 25.8 kcal mol^{-1} (leucine) to 29.2 kcal mol^{-1} (phenylalanine) also increase their E_a on including the fossil data, but the ΔE_a values range in a relatively narrow interval around $\pm$9.00 kcal mol^{-1}, and their kinetic models could be used with relative confidence in dating studies.

We dedicate this paper to the memory of the late Glenn A. Goodfriend, PhD, who helped us in an incredible way in the setup of our laboratory of amino acid racemization.

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